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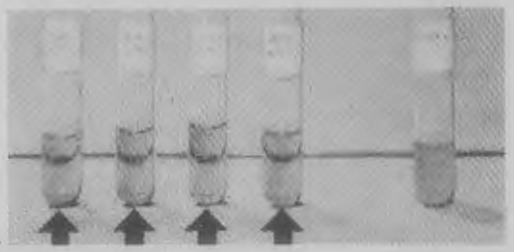

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SIMPLE TESTS FOR GAMMA GLOBULINS INCLUDING VISCOMETRY

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A short review of serum protein fractionation was given in a previous paper (Fischman, 1954). Some of the main points were as follows: 1. The advantages of the true albumin methods over the Howe fractionation were pointed out. There was no unanimous agreement in the literature at that time, whether or not the true albumin technique should replace the more conventional methods.

More recent textbooks seem to have decided in favour of the "true" fractionation.

2. Simple chemical methods of estimating globulin fractions were described.

While electrophoresis is universally used for fractionation of globulins, interest is being maintained in simpler and quicker estimations of gamma globulin alone, both in agammaglobulinaemia and in conditions with raised gamma globulin (GG). The present paper describes simple methods of GG. estimation other than the chemical methods referred to in the previous paper.

I. *VISCOMETRY*

The direct relationship between protein concentration and viscosity of a solution has been known for many decades. Serum viscosity (V.) estimation as a measure of total protein was a routine procedure. The albumin/globulin ratio was determined by simultaneous estimation of V. and the refractive index of serum. More recently a combination of V. and specific gravity measurements were utilised for the A/G ratio (Eastham, 1954). Plasma V. as an index of additive changes in fibrinogen and GG. level has been routinely estimated by many rheumatic clinics.

Lawrence (1950) introduced the fractional plasma viscosity estimation, to measure all main protein fractions, especially GG. and fibrinogen. (See also Whitby and Britton, 1953). This method consists essentially of measuring plasma V. first, then removing fibrinogen by the usual method, and measuring V. of serum. The difference gives the fibrinogen level expressed in relative V. units. GG. is estimated by removing GG. from serum using ammonium sulphate, and measuring the V. of the filtrate. The difference between serum V. and filtrate V. gives the GG. level.

This method is relatively simple, but has some disadvantages:

1. It requires estimation of V. of two different solutions.

2. Calculation is by difference. Error caused by this is mentioned by Lawrence.

3. Further arbitrary adjustment of V. value is necessary to allow for the considerable increase of V. of the serum filtrate, owing to the high concentration of ammonium sulphate.

In the present study it was attempted to eliminate some of the disadvantages mentioned above, by precipitating GG., redissolving it and estimating V. of the GG. solution directly. This procedure requires testing of one solution only and obviates necessity for calculation by difference and correction for salt content.

Technique

The technique of measuring viscosity has been described in a previous paper (Fischman, 1950).

The reagent for estimating gamma globulin contains 204 grams of ammonium sulphate and 34 grams of sodium chloride per litre. Pipette 10.8 ml. of the reagent into a 15 ml. strong centrifuge tube. Layer 1.2 ml. of serum on top of the solution. Mix by slow, repeated inversion for about two minutes. Centrifuge for 30 minutes. Carefully invert and drain on filter paper. Dissolve the precipitate in 1.2 ml. of physiological saline, and estimate the flowtime in the viscometer. The flowtime divided by flowtime of saline gives relative viscosity.

Comment

The salting out procedure of Wolfson, et. al. (1948) is applied in the method described above. This is claimed to give a better and quicker yield of GG. than other ammonium sulphate procedures. A final concentration of 1.39 M. ammonium sulphate is required. The final NaCl concentration is 40 gm./1000 according to Wolfson, and 30 gm. according to Popper, et. al. (Fischman, 1954). The quantity of serum used in viscometry is arbitrary and depends on the capacity of the viscometer. Whittington's meter needs less than 1 ml, while Lawrence's meter uses more than 2 ml. If a dilution other than the above is used, the concentration of the reagent has to be changed to give the required final concentration.

The normal value in a small series of forty healthy subjects ranged from 10 to 19 (1.10-1.19 relative viscosity units). In conditions with raised GG., values ranging from 20 to 45 were found. These levels are comparable with those of Lawrence (1950), they tend however to be slightly lower. This may be due to different technique and/or different viscometer. Values obtained by different meters vary somewhat and methods of calibration to obtain uniform values are not quite satisfactory. It seems desirable to establish normal and abnormal ranges for the

particular apparatus used, as there is no standard meter used by all workers.

Conversion into gm./100 ml.

To express results in gm./100 ml. instead of V. units, two procedures may be adopted:

1. A graph may be prepared, based on simultaneous estimations of GG. by V. and a recognised chemical method. Lawrence did not attempt to express values in gm. percent.

2. GG. of serum expressed in V. units may be correlated with V. levels of pure GG. solutions of known concentration. This seems theoretically the best method, but is nevertheless beset with difficulties. Lawrence mentions that his serum GG. values are considerably higher than V. levels obtained for pure GG. solutions of similar concentration.

Estimation of V. of a pure GG. solution is dependent on availability of pure GG. V. of one sample of a 16 percent GG. solution (Lederle) diluted 1:10 was found to be about 15 percent lower than V. of a serum with a GG. concentration of 1.6 gm. percent in the present study. Several known and unknown factors make it difficult to use pure GG. for calibration purposes. Further work combined with electrophoretic checks is required to clarify this matter. There is no detailed work in the literature on viscosity of pure GG. solutions. For practical purposes workers seem to prefer the simpler procedure of expressing concentrations in V. units only.

II. *IMMUNOLOGICAL METHODS*

An extensive literature exists on estimation of protein fractions by immunological methods. Some of these procedures are mainly research tools, and fairly complicated. These techniques are either purely immunological, or combine immunological techniques with electrophoresis. These methods play an increasingly important role in characterisation and separation of globulin fractions, going beyond the possibilities attainable by electrophoresis only.

Simple estimation of gamma globulin is also possible, using serological technique. One such method is that of Wiener (1955). This is based on the fact that gamma globulin can inhibit the reaction of the antiglobulin serum. Serial dilutions of the serum to be tested and a normal serum are made. Antiglobulin serum is added, and a Coombs test performed on cells sensitised with anti D serum. Normal serum, i.e. serum with a normal gamma globulin content inhibits the test up to a dilution of 1: 128, while in the absence of GG. in a serum even the undiluted specimen causes

no inhibition. From the ratio of the titre of the patients' serum and normal serum the concentration of GG. may be calculated. More technical details are given in the original papers.

SUMMARY

Interest is being maintained in simple methods of estimating serum gamma globulins. A method of estimation by viscometry is described. Reference is made to simple serological and chemical methods.

ACKNOWLEDGEMENT

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METHODS FOR ISOLATION OF PATHOGENIC STAPHYLOCOCCI FROM VARIOUS SOURCES

JOY SORENSEN
(National Health Institute)

In the course of investigations being carried out at the National Health Institute which call for the isolation of pathogenic staphylococci from different sources such as nasal swabs, lesions, faeces, blankets and dust, it has been necessary to compare and evaluate the efficiency of different methods of isolation. The purpose of this paper is to record and discuss our experience with methods and media now being used at this Institute for this purpose.

As there is ample evidence that the most convenient and reliable single test for pathogenicity in staphylococci is that of coagulase production, this criterion is used in investigations carried out here. As no differential medium has yet been found which will satisfactorily replace the Fisk (1940) method for testing coagulase production, this is the method of choice.

0.5 ml. of a 1:10 dilution of pooled human plasma plus 5 drops of a 6 to 18 hr. broth culture, incubated in a 37°C waterbath, is the adaption of the Fisk method used here. Failure of the majority of coagulase positive staphylococci to clot in one hour under these conditions, indicates that the plasma is not suitable. Plasma left at room temperature rapidly deteriorates and should be kept in the refrigerator when not in use. The inspection of tubes every quarter hour to see if clots have formed is not recommended as the clots are broken up and the final reading difficult to make. The first reading is made at one hour, and then at hourly intervals for six hours.

To determine the time necessary for clot lysis, two hundred and fifty staphylococcal cultures chosen at random from specimens received were tested for coagulase production and left in the incubator after clots had formed. The shortest time taken for any clot to dissolve was five hours.

Contaminated cultures often give false negative results. The contaminants, usually Gram negative bacilli and non-haemolytic streptococci apparently inhibit the growth of the staphylococci.

This is probably the reason for reported anomalies with the coagulase test such as the apparent change from coagulase positive to coagulase negative and vice versa. The best method of picking off a single colony to obtain a pure culture is to use a straight wire and only touch the top of the colony. At the National Health Institute all cultures received which are coagulase

negative are put through a salt broth and then plated, but it is often impossible to recover coagulase producing staphylococci. As these contaminated cultures delay phage typing procedure, bacteriologists referring cultures of staphylococci to this Institute should ensure that the cultures are pure.

Isolation from Nasal Swabs and Lesions

As it has been shown that a third to a half of the population are nasal carriers of coagulase positive staphylococci, any method used for nasal swabs should be capable of staphylococcal isolations of this order.

The usual method for examining swabs from either noses or lesions is to plate on blood agar and incubate the swab in nutrient broth. Blood agar is generally regarded as the most satisfactory medium for direct plating as it grows other pathogenic bacteria as well as staphylococci. It has been found here, that 10% salt broth is preferable to nutrient broth as it inhibits the growth of Gram negative bacilli and other bacteria which often mask the staphylococci present. Even better results have been obtained using Hartley's digest broth plus 10% salt. It is important to incubate salt broths 48 hours, as the high concentration of salt seems to have a temporary inhibitory action on the staphylococci. Salt broth is a suitable medium for the isolation of staphylococci from nasal carriers. The nasal swabs are put directly into salt broth, incubated 48 hours and then plated on blood agar.

Isolation from Blankets

The most satisfactory method in our experience for isolating staphylococci from blankets is the sweep plate method of Williams reported by Blowers and Wallace (1955). This method was used by these authors to test the efficiency of cirrasol O.D. as a disinfectant for blankets.

A petri dish face down is stroked vigorously, three or four times, for about eighteen inches across the blanket to be tested. The advancing edge of the plate throws up dust and fluff on to the medium. The medium used by Blowers and Wallace was nutrient agar containing 0.75% perminal C.O.L. which was intended to neutralise the cirrasol remaining in the blanket after washing. However, the two batches of perminal tested here proved to be inhibitory to staphylococci and other organisms. Thus when a large drop of a one in a million dilution of a 4 hour broth culture of staphylococci was put on a plain nutrient agar plate and on plates of nutrient agar containing 0.2%, 0.1%, 0.075%, 0.05% perminal, and incubated overnight at 37°C, the nutrient agar plate grew 46 colonies of staphylococci while the plates with varying amounts of perminal added grew only an average of 6 colonies.

Average total colony counts of blankets also showed a marked increase when nutrient agar plates were used without the addition of perminal. The average colony count on sixteen blankets tested with nutrient agar plates with perminal added was 109 before washing and 3 after washing. The average colony count on sixteen blankets using nutrient agar was 1,773 before washing and 23 after washing.

The medium used at the National Health Institute for all blankets is Barber and Kuper's medium which is nutrient agar (pH7.4) and 0.01% disodium phenolphthalein phosphate (P.P.A.). This method depends on the phosphatase breaking down the disodium phenolphthalein phosphate to phenolphthalein which becomes pink on exposure to ammonia. Phosphatase production in staphylococci is correlated with coagulase production. The blanket plates are incubated overnight at 37°C and next morning each plate is exposed to ammonia fumes. A convenient way of doing this is to place 0.880 ammonia in a small basin at the bottom of a 120 mm. desiccator. An ordinary size petri dish (9 cm) fits face down over the opening to the lower compartment. The phosphatase positive staphylococci turn bright pink on exposure to ammonia, sporing bacilli also turn pink, but are easily distinguished by colony size, but unfortunately some coagulase negative staphylococci react though they are generally a paler shade.

When 90 phosphatase positive colonies were picked off blanket plates 77 proved to be coagulase positive and with another batch of blanket plates 75 phosphatase positive colonies were picked off and 68 proved to be coagulase positive.

Although a worker experienced with this medium may become very proficient at deciding which shade of pink is associated with coagulase positive colonies, it is doubtful whether this method could ever be completely reliable. However, P.P.A. plates are excellent for isolating coagulase positive staphylococci when other bacteria are present, provided that the colonies so isolated are checked by the tube coagulase test. When testing blankets with colony counts in the hundreds it is tedious and time consuming to differentiate coagulase positive staphylococci when agar plates are used without the addition of P.P.

Isolation From Dust

Two methods were tried for isolation of staphylococci from dust. First an Ingram's syringe was used for blowing dust on to the surface of a P.P.A. plate. Although this proved satisfactory it required two workers, one to handle the syringe and the other to hold the plate. An equally efficient method is to use cotton wool swabs and salt broth. The swabs are dipped in salt broth

and rubbed several times across the surface to be tested. These are put in salt broth, incubated 48 hours and plated on blood agar plates or P.P.A. plates. It is very important to incubate salt broths 48 hours. This was shown when fourteen samples of dust were taken from a ward and the salt broths plated at 24 hours and 48 hours. After 24 hours coagulase positive staphylococci were isolated from 5 salt broths, after 48 hours twelve salt broths were positive. Similar results were obtained from further tests.

Isolation From Faeces

Salt broth is also used for isolating staphylococci from faeces. Again the most reliable results are obtained if salt broths are incubated 48 hours and then plated on blood agar or P.P.A. Direct plating on blood agar is to be recommended especially when staphylococcal enteritis is suspected. In some hospitals direct plating on blood agar is included as part of the general routine for all faecal specimens received for bacteriological examination. Tellurite glycine agar (Zebovitz, Evans, Niven, 1955) was also tested. As it is an inhibitory as well as a differential medium it was hoped that it would be useful for isolating staphylococci from faeces. However, the medium is difficult to prepare as the amount of tellurite to be added has to be determined for each batch of tellurite, and it was decided that the two media mentioned above gave as good results as the tellurite glycine agar.

SUMMARY

Some of the methods used in this Institute for isolating staphylococci are described. Of the media, 10% salt broth and phenolphthalein phosphate agar have proved the most useful.

ACKNOWLEDGEMENT

I wish to thank Dr. J. D. Manning, Director, and Mr S. W. Josland, Chief Bacteriologist, of the National Health Institute, for their helpful advice and criticism in the preparation of this paper.

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A RELIABLE METHOD OF ANAEROBIOSIS

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Anaerobic plate culture is too often considered to be a troublesome procedure by many diagnostic laboratories and as a result this method of examination may not be used as often as is desirable.

An attempt has been made in this laboratory to develop an easy and efficient arrangement for anaerobic plate culture and it is thought that the method now in use here may be of interest to other laboratories in this country.

APPARATUS AND METHOD: The method used follows that described by Hayward (1945) who used the palladiumized asbestos capsule described by Wright to catalize the combination of oxygen with hydrogen in a suitable container such as a McIntosh-Fildes anaerobic jar.

Some organisms, especially members of the genus Bacteroides, require carbon dioxide and it is desirable in anaerobic plate culture to incorporate approximately 5% carbon dioxide in the atmosphere.

Container: Because the amount of anaerobic plate culture work being done was too great for the available McIntosh-Fildes jars it was found necessary to obtain a larger container.

The details of a satisfactory conversion of a 16 pint "Hawkin's" pressure cooker into a useful anerobe jar may be of interest. The cooker lid was inverted and fitted with two gas taps sealed with washers. The centre tubular steam vent of the cooker lid was plugged with solder and rescrewed into the top of the lid in the inverse position. The main carrying handle was shortened. The removable lid handle was extended by a piece of bar steel hooked at one end to fit under the small carrying handle and a small pin to fit into the inverted steam vent was welded on to the under surface of the metal handle shaft. When the handle is clamped into position with the pin engaged in the steam vent tube the resulting pressure holds the lid tightly in place. The completed jar with lid in position is shown in Fig. 1.

Wright's Capsule: Directions for the preparation of this capsule as given in Hayward's paper are given in the appendix.

Supply of Carbon Dioxide and Hydrogen: The carbon dioxide and hydrogen supplies are obtained from cylinders, through reducing valves passing into anaesthetic re-breathing bags and thence to the control panel, where a single supply line reaches the jar through a Y piece. The control panel is shown in Fig 2.

Evacuation of Jar: To reduce the risk of explosion it is

Fig. 1: Showing lids and handles of pressure cookers before and after modification. The bodies of the cookers are of different models but lids and handles were identical.

essential to evacuate the jar, the minimum vacuum required being 3/5th of an atmosphere. A water vacuum pump is employed for this purpose. Flooding of the jar is avoided by use of a water trap, situated between the pump and the vacuum tap on the control panel. A gauge is included between the jar and the vacuum tap so that the degree of vacuum and the approximate gas intake can be assessed.

Method: The following instructions are carried out in setting up the anaerobe jar.

1. Heat capsule and put into clip on lid. Inflate re-breathing bags.
2. Put plates, etc., into jar and seal the lid.
3. Attach tubes and open both taps on jar.
4. Evacuate to 25 inches of mercury and turn off vacuum tap.
5. Run in carbon dioxide until gauge reads 25 inches of mercury.
6. Open hydrogen tap and leave five minutes.
7. Close hydrogen tap and both taps on lid.
8. Incubate.

DISCUSSION: This method has been in use here for the past year and the additional numbers of anaerobic streptococci and Bacteroides strains isolated, together with the ease of grow-

ing species of Clostridia, have justified our confidence in the use of this technique.

APPENDIX

The capsule known as Wright's capsule is made as follows: one gram of palladium chloride is dissolved in 10 ml. of distilled water containing a few drops of concentrated hydrochloric acid. The palladium chloride is not completely soluble in the water and acid but this does not seem to matter, the "solution" may be warmed a little to encourage it to dissolve further. Then 1.5 gm. of asbestos wool is soaked thoroughly in the preparation and dried in the incubator. The asbestos is teased out and heated in a smoky candle flame until black, then a blow-pipe flame until the black goes. A fine divided deposit of palladium then covers the asbestos fibres.

The catalytic activity of this preparation may be tested by directing a fine jet of hydrogen on to it. The asbestos should warm up and glow, igniting the jet of hydrogen in a few seconds. This amount of catalyst is sufficient for six capsules. Each capsule is made by spreading the palladiumized asbestos loosely in a thin layer 1½ inches square on one half of a 1-5/8th in. x 3¼in. sheet of 30-40 mesh copper gauze, folding over the unoccupied half of the gauze and closing the capsule by turning in the edge of the two layers, with a second fold of 1/8 in. on the three open sides.

These capsules may become inactive from two causes. First, a deposit of moisture on the palladiumized asbestos inhibits its catalytic activity. If the capsule is heated moderately strongly in the outer cone of a Bunsen flame the moisture is driven off and the activity is restored. Such heating is needed frequently and may be done as a routine every day, the capsule cooling as the jar is filled with cultures. The second cause of inactivity is the formation of inactive palladium sulphide from hydrogen sulphide evolved by cultures in the jar. This inactivation can be reversed only by opening the capsule and re-roasting the palladiumized asbestos. A number of spare capsules should be kept so that if one is found to be inactive it can be quickly replaced.

The outer cone of the flame is recommended for the daily heating to drive off moisture, as the inner blue cone contains unburnt gasses including sulphides, which may poison the capsule.

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Fig. 2: The apparatus ready for use.

NOTES ON CO_2 DEPENDENT STAPHYLOCOCCI

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Dwarf colony variants of staphylococcus have been reported on a number of occasions. These have been produced by growing staphylococci in media containing inhibitory substances of various kinds including antibiotics or have been isolated from patients treated previously with antibiotics or sulphonamides. Such organisms were reported by Sherris (1952), Goudie and Goudie (1955), and Thomas (1955). These organisms gave dwarf colonies on primary culture aerobically but normal large colonies in the presence of 10% CO_2.

Two similar organisms have been isolated in this laboratory.

CASE 1. A child of seven years was admitted to hospital on 25.6.56 with otitis media in the left ear. The ear had been discharging for six weeks. It was subsequently learned that the condition had been treated with aureomycin previous to admission.

Direct examination of a swab from the ear showed pus, numerous staphylococci and a few Gram positive bacilli.

Cultures on blood agar gave a growth of a staphylococcus (very small colonies after 48 hours) and a streptothrix.

The staphylococcus colonies were described as pin point colonies and were scarcely visible without a lens.

When subcultures on blood agar were incubated in 10% CO_2, in a "candle tin" and on tubes of Loeffler's serum (corked), colonies of normal size were obtained. When the growth was heaped up with a platinum loop it gave some yellow colour and was coagulase positive by slide and tube techniques. Sensitivity tests by the ordinary disc method showed the following:—

Penicillin	Achromycin	Terramycin	Streptomycin	Aureomycin
+	++	++	++	++

Chloromycetin	Crystamycin	Erythromycin.
+	+	++

+ means sensitive.

The organism grown in 10% CO_2 gave the following reactions:—

Lactose	Glucose	Sucrose	Maltose	Mannitol
+	+	+	+	+

+ means acid.

Colonies on human blood agar showed zones of haemolysis.

Methyl red	+	Gelatine: Saccate liquefaction in 10 days.
V.P.	—	Broth: Good growth.
Citrate	—	Litmus Milk: Acid.
Indole	—	
Catalase	+	
Urease	+	

Grown aerobically on blood agar with a strain of staphylococcus of normal colony size, the organism exhibited satellitism. There was no visible growth aerobically in broth.

Subcultures grown anaerobically gave only small colonies.

One month after isolation subcultures of the organism still gave the small type of colony aerobically but it was noted that if a heavy inoculum was used from the large type of colonies, the large type of colony growth was obtained aerobically. However, if the organism was suspended in peptone water till just opalescent, subcultures from this aerobically gave discrete pin point colonies. The large type of colony was always given in 10% CO_2.

Similar results were given ten months after isolation.

Phage typing. This was done at the National Health Institute and the phage type was found to be 3B/3C.

CASE 2. An adult male aged 33 years, admitted to hospital on 31.8.55 with poliomyelitis, developed chronic cystitis. His urine contained pus and at different times over about eighteen months the following organisms were isolated—Pseudomonas pyocyanea, Streptococcus faecalis, a coliform bacillus and Staphylococcus aureus. The latter grew aerobically and was coagulase positive.

On 19.9.56 it was found that he had an infection with a staphylococcus which was seen in large numbers in the direct smear, but which failed to grow aerobically. This suggested that the organism might grow in an atmosphere of 10% CO_2 and this was found to be the case and on human blood agar the colonies were of normal size. Aerobically at this stage there was no growth in 48 hours on subculture with a thin inoculum, but with a thick inoculum pin point colonies scarcely visible without a hand lens were seen.

When grown aerobically in the vicinity of a normal staphylococcus, the organism showed satellitism, but even then the growth was weak in 48 hours.

The organism was described as Staphylococcus aureus, but the pigment was rather weak and the zones of haemolysis on human blood agar plates were weak. Pigment was lost in later

subcultures. Coagulase tests were positive by slide and tube techniques. Anaerobic cultures gave no growth on blood agar after 42 hours.

30.5.57. Subcultures on agar slopes. With cotton wool plugs there was no growth in 18 hours, but a fair growth of small colonies in 42 hours.

On corked agar slopes, growth was good after 18 hours although the colonies were somewhat small.

Sensitivity tests on blood agar by the ordinary paper disc method showed the following:—

Penicillin	Aureomycin	Achromycin	Terramycin	Streptomycin
—	++	++	++	++

Chloromycetin	Erythromycin.
++	++

\+ means sensitive.

The patient had been treated according to the infection at the particular time with furadantin, polymixin B, erythromycin, again polymixin B and chloromycetin, so that again we find a dwarf colony type of Staphylococcus aureus, arising after antibiotic treatment.

The CO_2 dependent staphylococcus was reported a number of times till 7.11.56 when an aerobic Staphylococcus aureus was reported. On 14.11.56 it was reported as "mucoid". Again on 10.1.57 the organism failed to grow aerobically but on 4.3.57 grew well aerobically.

The patient had been discharged on 3.5.56 but had attended the Outpatients' Department still with chronic cystitis caused by a pure culture of a Staphyloccus aureus which was present microscopically when the last specimen was examined on 30.4.57.

Grown in 10% CO_2 the dependent organism gave the following reactions:—

Methyl red	+	Gelatine: Failed to grow.
V.P.	—	Broth: Fair growth.
Citrate	—	Litmus Milk: Acid.
Indole	—	
Catalase	+	
Urease	—	

Lactose	Glucose	Sucrose	Maltose	Mannitol
+	+	+	+	+ (slow)

\+ means acid.

The organism was not phage typed.

DISCUSSION:

Topley and Wilson, 4th edition, quoting Gladstone et alii (1935) states of Staphylococcus aureus that no growth occurs in

complete absence of CO_2. Obviously for most strains there is sufficient in the atmosphere and media. In the case of dwarf colony strains, to give normal sized colonies, extra CO_2 must be supplied, e.g., by means such as acid and bicarbonate or "candle tin" or apparently by heavy inoculum in subcultures or by the presence of a normal culture when satellitism is seen, or by complete closure of a culture tube by a cork when the CO_2 content will build up.

The obvious practical conclusion is that the staphylococcus may fail to grow on culture if a plate is not incubated in extra CO_2.

ACKNOWLEDGEMENTS

I wish to thank the Medical Superintendent, Dr. McNickle, and the Pathologist, Dr. D. Allen, for permission to publish these notes.

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THE UBIQUITOUS T.N.P.N.

C. E. FLEMINGHAM
(*Whangarei Hospital*)

We have noticed in this laboratory over the past few years that the more methods there are for the estimation of T.N.P.N.s, the wider is the range of figures obtainable on the one specimen. For example, using trichloracetic acid (1) as the protein precipitant, gave higher results than did using tungstic acid (2), which in turn gave slightly higher figures than did a micro-Kjeldahl technique. Perchloric acid (3) gave results akin to micro-Kjeldahl, while sulphosalicylic acid and copper tungstate gave results that were approximately 10% lower than those obtained with micro-Kjeldahl. This latter observation was understandable, as glutathione and ergothionine, present in blood in the range 5-10 mgm./100 ml., form insoluble copper compounds with copper, and are therefore precipitated out. N.P.N.s performed on tungstic acid precipitated specimens, using unlaked blood in sodium sulphate, gave low results equivalent to those performed on the same specimens using copper tungstate as the protein precipitant, thus confirming that the low results with the latter could be due to the copper compounds of glutathione and ergothionine, as these substances are not liberated from unlaked cells.

This did not explain the difference between the trichloracetic acid, tungstic acid, perchloric acid, and sulphosalicylic acid methods, nor did it explain occasional turbid, nesslerised end-points. As a result, an attempt was made to investigate the subject of T.N.P.N.s.

In all the methods, with the exception of the perchloric acid one, 50% sulphuric acid containing 1% selenium dioxide was used as the acid digestion mixture, as this prevents complete evaporation during digestion, if overcooking takes place, and hence eliminates the possibility of the glutinous mess sometimes achieved, and enables heating to continue for 3-4 minutes after clearing, thus ensuring complete digestion.

Effect of Heat

As stated above, it was sometimes found that our nesslerised endpoints were turbid, although the possibility of contamination with acetone fumes could be eliminated. As digestion was done on a converted electric heater, the possibility of insufficient heat arose, and so samples were simultaneously estimated in duplicate on the electric heater, and on Bunsens turned "low", "medium", and "high". All methods were treated in this way, and varying lengths of time of heating were allowed following clearing, with

the results as shown in Figure 1. These results are typical of the 24 bloods treated in this manner, regardless of the method used.

Source of Heat	Minutes of Heating continued after clearing.											Turbidity
	0	5	10	15	20	25	30	45	60	90	120	
Electric	41	41	41	41	38	36	36	32	32	28	20	Present in 20%
"Low" Bunsen	41	41	41	37	35	33	32	32	32	18	6	Absent
"Medium" Bunsen	41	41	35	14	3	2	0					Absent
"High" Bunsen	41	25	4	0								Absent

Figures represent mgms. of N.P.N./100 ml.

Figure 1

We can only assume that power fluctuations causing even lower heat than usual on the electric heater, were the cause of the turbidity. At this stage we abandoned electric heat, and were not troubled by turbidity again.

No attempt was made to measure the heat of the Bunsens, but as it was apparent that too much heat for too long caused a dramatic loss of ammonium salt, we continued using "low" Bunsen heat, continued for 3 minutes after clearing. The loss of ammonia on excess heating remained an interesting though unexplained phenomenon, in view of the fact that sulphuric acid decomposes into sulphur trioxide and water vapour (the recombining of which forms the characteristic "dense white fumes") at 340°C, and ammonium sulphate decomposes at 280°C!

In an attempt to find the lost ammonia after excess heating, the fumes driven off during digestion were collected into water. In all cases, the resultant solution was nesslerisable, with dense turbidity, but contained no ammonium radicle, unless heat had been continued until such time as ammonia loss was apparent in the actual N.P.N. We can only assume that where no ammonia loss took place, and no ammonium radicle was detected in the collected fumes, that serum acetone was responsible for the colour and turbidity produced on nesslerisation. The collected fumes did not give a positive result with Rothera's test, which will not detect a 1/40,000 dilution of acetone, but a 1/80,000 dilution of acetone will however react with Nessler's, producing turbidity and yellow colour, and the heat applied during digestion would drive off the small quantity of acetone normally present in blood. However, where excess heat had been applied, ammonia was detectable by steam collection and subsequent titration.

Effect of Protein Precipitant

Fifty bloods were examined, each by precipitation with trichloracetic acid, tungstic acid, and copper tungstate, followed by digestion and nesslerisation, and by the micro-Kjeldahl technique.

Assuming the micro-Kjeldahl technique using tungstic acid precipitation to be the true N.P.N. value, the following pattern evolved.

	Nesslerisation	Micro-Kjeldahl
Trichloracetic acid	Approx. 10% high	True value
Tungstic acid	Approx. 3% high	True value
Copper tungstate	Approx. 10% low	Approx. 10% low

Sulphosalicylic acid as a protein precipitant was abandoned, as it gave results similar to copper tungstate, and presumably was precipitating some nitrogenous substances, as was copper tungstate, i.e., ergothionine and glutathione. Copper tungstate was also abandoned at this point, and was replaced by perchloric acid (3), which gave results 3% lower than did tungstic acid. In other words, it agreed with the micro-Kjeldahl technique.

Paper chromatography of the filtrates before digestion did not reveal any detectable substance in the trichloracetic acid method, which was not present in the tungstic acid one.

Results obtained to this point seemed to indicate that the various protein precipitants interfered with the Nessler's reagent. This now seems to be the case.

We now find that if a blood is estimated by each of the three methods, trichloracetic acid, tungstic acid and perchloric acid, and if blanks consisting of a similar quantity of filtrate to that digested, plus a similar quantity of acid digestion mixture, with sufficient water to make the required volume, and Nessler equal to that used in each method, are subtracted from the optical density reading of the unknown, and a blank of water, acid digestion mixture and Nessler is subtracted from the optical density reading of the standard, the result with all methods is similar, and agrees with that obtained by the micro-Kjeldahl technique.

As the perchloric acid technique uses this acid both as a protein precipitant and as a digestant, only one blank is necessary, and suffices for both Test and Standard. As it also eliminates one step in the procedure, it is now our method of choice.

As we are no longer supplied with gas from a town supply, but rely on acetylene, we tried a Gallenkamp electrically operated

Kjeldahl heater, in an endeavour to economise on acetylene, and find that it is completely satisfactory, presumably because of a higher heat output than our original electric heater.

SUMMARY

The effect of heat and adequate blanks are discussed, with their influence on the "actual" T.N.P.N.

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INTERMEDIATE EXAMINATION FOR HOSPITAL LABORATORY TRAINEES

April 30 and May 1, 1957
NATIONAL HEALTH INSTITUTE, WELLINGTON
Examiners: Dr. J. T. O'Brien, Mr N. J. Ellison

WRITTEN PAPER

Time allowed: 3 hours. April 30, 9.30 a.m.-12.30 p.m.

1. Write *brief* notes on the following:—
   (a) Hydrogen ion concentration.
   (b) Achlorhydria.
   (c) Pasteurisation.
   (d) Antibody.
   (e) Coombs' test.

2. What are reticulocytes? How is their number assessed and what information is gained from this assessment?

3. Describe methods for the laboratory diagnosis of:
   (a) Undulant fever.
   (b) Diphtheria.
   (c) Glandular fever.
   (d) Gonorrhoea.

4. Given a sample of urine how would you determine the following:
   (a) Urea (quantitative).
   (b) Sugar (quantitative).
   (c) Albumin (quantitative).
   (d) Acetone (qualitative).
   (e) Bile (qualitative).

5. You are asked to provide blood for an exchange transfusion of an erythroblastotic baby. You are provided with oxalated and clotted cord blood. Detail your procedure.

PRACTICAL EXAMINATION

Tuesday, April 30, 2 p.m.-5 p.m.

Wednesday, May 1, 9 a.m.-10 a.m. (for completion of cultures)

SECTIONS 2, 3, and 4 must be completed on Tuesday.

SECTION 1:

(a) Report, including culture, on the urine A. (Pus cells, red cells, phosphates, albumin, coliform bacilli.)

(b) Report, including culture, on the swab B. (coliform bacilli, streptococci).

(c) Using the disc method carry out sensitivity tests on the culture C against the antibiotics penicillin, erythromycin and aureomycin.

SECTION 2.

(a) Determine the total non-protein nitrogen of blood specimen D. Give the normal range.

(b) Test urine E for sugar and acetone qualitatively.

(c) Test faeces F for occult blood.

SECTION 3:

Report briefly on slides labelled 1 to 10.

1. Hypochromic anaemia.
2. Erythroblastosis.
3. Neutrophil leucocytosis.
4. Eosinophilia.
5. Lymphatic leukaemia.
6. Vincent's organisms.
7. Meningococcus.
8. Monilia.
9. Actinomyces.
10. B. subtilis.

SECTION 4:

(a) Carry out leucocyte count, differential leucocyte count, and reticulocyte count on blood G.

(b) Prepare two Pasteur pipettes and two throat swabs.

ORAL EXAMINATION

The oral examination will commence at 11 a.m. on Wednesday, May 1.

The successful candidates were:—

CLARKE, Y. A. (Auckland)
GARNHAM, F. C. (Napier)
HILL, G. J. (Auckland)
HOLLOWAY, J. E. (Auckland)
KIRKER, J. E. (Wellington)
KOBERSTEIN, N. M. (Auckland)
McCARTHY, M. D. (Auckland)
MILLER, T. E. (New Plymouth)
NIXON, A. D. (Auckland)
PRENTICE, P. J. (New Plymouth)
ROY, D. (Auckland)
SMITHERAM, C. H. (Auckland)
STYLES, J. A. (Napier)
SHREEVES, K. J. (Rotorua)
YEARBURY, B. J. (Auckland)

All candidates who presented themselves for this examination were successful.

FINAL QUALIFYING EXAMINATION FOR CERTIFICATE OF PROFICIENCY IN HOSPITAL LABORATORY PRACTICE

26th-28th FEBRUARY, 1957

NATIONAL HEALTH INSTITUTE, WELLINGTON

Examiners: Dr. S. Hills (Auckland), Dr. C. B. Pearson (Christchurch), Dr. M. G. Somerville (Tauranga)

THEORETICAL PAPER

Tuesday, February 26th, 9.30 a.m.-12.30 p.m.

Time allowed 3 hours.

Each question carries the same marks. Answers should be neatly set out and concise.

1. Briefly describe an example of a Complement Fixation Test, an Agglutination Test and a Precipitin Test as commonly used in Hospital Laboratory Diagnosis.

2. Give an account of the Neisseria Group of Organisms with the methods of Laboratory Diagnosis of the more common members.
3. Describe one method of demonstrating L.E. cells in peripheral blood.
4. How would you perform:—
   (a) A reticulocyte count.
   (b) A platelet count.
   (c) A direct Coombs' test.
5. Describe the instruments available for measuring the optical density of solutions as applied to biochemical estimations. What are their advantages and disadvantages?
6. Write concise notes on any 5 of the following:—
   Milli-equivalents, Urease tablets, Blank, Topfers indicator, Thymol Turbidity Test, Pandys Test, Formaldehyde stable acid phosphatase.

BIOCHEMICAL PRACTICAL EXAMINATION

(3 hours)

1. Perform an alkaline phosphatase determination on the serum labelled 'A.' Clearly put down on paper the principle of the test and how you performed it and arrived at your result.
2. Perform a calcium estimation on the serum labelled 'B.' Clearly put down on paper the principle of the test and how you performed it and arrived at your result.
3. You are provided with a routine non-catheter urine specimen from a man. Perform the usual routine biochemical tests as well as examining the deposit and tabulate your results. Also test the urine for urobilinogen.
4. You are provided with a small sample of faeces from a man with anaemia of unknown cause. He has been off meat and green vegetables for three days. Perform an occult blood test. Name the method you used.
5. Six spots are provided labelled 1-6. Write brief notes on each.

(Among the spot tests provided were: uric acid tube, perchloric acid, molybdic acid, sodium barbitone, barbituric acid, phenol, glycine, protein digestion tube, sodium tungstate and sulphuric acid, balance rider, sintered glass filter, methyl red, Babcock tube, balance scale, Nessler's reagent, blue optical filter, sodium metabisulphite, electrophoretic strip, weighing bottle, soluble starch, trichloracetic acid, Evans blue dye, octyl alcohol, selenium barrier layer cell.)

BACTERIOLOGY—PRACTICAL I

(1½ hours allowed on each of two successive days)

1. Identify the organism on plate 1, stating steps taken.
   The identification should be completed on the second day.
   (S. cholerae suis or S. bovis morbificans.)
2. Identify the organism on plate 2, stating steps taken.
   The identification should be completed on the second day.
   (C. diphtheriae or pneumococci.)
3. Identify the "spots" provided.

(Among the spot tests provided were: ova of Ascaris lumbricoides, macroconidia of Microsporum canis, actinomyces, C. diphtheriae, bone marrow biopsy set, sequestric acid, Trypansoma, defibrinator, microscope light, leprosy (stained section), hydatid hooklets (sectioned), N. gonorrhoeae, film of peripheral blood (over-stained with Leishman's stain), section stained H and E (not dehydrated.)

BACTERIOLOGY—PRACTICAL II

1. Complete questions 1 and 2 carried over from previous day.
2. Identify the "spots" provided.

HAEMATOLOGY AND HISTOLOGY I

(1½ hours allowed on each of two successive days)

1. The blood "A" was withdrawn at 11 a.m. today. Carry out:—
   (a) A haemoglobin estimation.
   (b) A platelet count.
   (c) A total white and differential count.

   The P.C.V. is 42%. What is the mean cell haemoglobin concentration?
2. Section "P" is a paraffin section. Stain it with H. and E. and mount it.

HAEMATOLOGY AND HISTOLOGY II

1. The serum "B" is from a patient requiring transfusion. Are the cells "X" and "Y" compatible? Do a slide test only and list what further tests you would carry out if you were doing a cross-match in your own laboratory.
2. "W" is a paraffin section from a prostate. Do a Van Giesen stain and mount it.

ORAL EXAMINATION

Subjects discussed at the Oral Examination were:

Leprosy, haptens, haemolytic streptococci, pathogenicity of staphylococci, plague and Pasteurella pestis, toxins and toxoids, immunity, Ehrlich's reagent, Topfer's reagent, acid and alkaline phosphatase, uses of para dimethyl benzaldehyde, types and principles of colorimeters, what to do with plain and oxalated cord blood from case suspected of having erythroblastosis, bleeding times, prothrombin times, processing of sections, stains for iron pigments, methods of haemoglobin estimation, normal values of methods discussed.

Candidates who passed were:

Mr W. ALDRIDGE (Wellington)
Mr J. C. BEATTIE (Auckland).
Miss M. F. BELL (Wellington)
Miss A. D. DUNNACHIE (Lower Hutt)
Miss M. P. EAGLE (Wellington)
Miss M. M. EALES (Christchurch)
Mr A. FISCHMAN (Auckland)
Mr D. W. FITZGERALD (Timaru)
Mr R. H. J. JONES (Auckland)
Miss S. A. JURY (Auckland)
Miss M. A. KENNEDY (Waikato)
Miss D. G. LUDBROOK (Auckland)
Mr J. G. MEREDITH (Auckland)
Mr R. W. SMAIL (Southland)
Mr B. N. SMITH (Timaru)
Miss J. B. SPEDEN (Christchurch)
Mr D. M. TAYLOR (Auckland)
Miss H. E. WATT (Wellington)

All candidates who presented themselves for this examination were successful.

ABSTRACTS

MANUAL OF AMERICAN SOCIETY OF CLINICAL PATHOLOGISTS WORKSHOP ON GLUCOSE

F. William Sunderman et al.
Amer. J. clin. Path., **26,** 1355, 1956

This is the second of what is to be an annual activity of the American Society of Clinical Pathologists. This year glucose has been chosen as the object of consideration. The subject is considered under various sections, namely:

(1) General information.
(2) Measurement of glucose (micro and macro).
(3) Identification of reducing substances including reactions with Benedict's qualitative reagents and with yeast; paper chromatography; reactions of sugars with phenylhydrazine; polarimetry; essential pentosuria.
(4) Gravimetric calibration of pipettes.

SOME OBSERVATIONS ON INFECTIOUS MONONUCLEOSIS IN OTAGO

N. P. Markham
N.Z. med. J., **55,** 49, 1956

Fifty-four serologically positive cases of infectious mononucleosis occurring in the Otago district over a four year period have been studied and details of age and sex distribution and seasonal evidence are given.

It was found that cases of infectious mononucleosis in the acute phase, had a definite disease pattern with regard to clinical, haematological and serological features. Details of differential white cell counts together with titres obtained at different periods are given.

Epidemic and sporadic forms of infectious mononucleosis are discussed and the importance of the differential sheep cell agglutination test is stressed.

A SIMPLIFIED TECHNIQUE FOR DIFFERENTIATION OF GROUP A (HUMAN PATHOGENIC) HAEMOLYTIC STREPTOCOCCI FROM OTHER GROUPS

L. Kirschner and T. Maguire
N.Z. med. J., **55,** 71, 1956

The authors report on a method for the differentiation of Group A (human pathogenic) beta haemolytic streptococci. This is a method first introduced by Maxted and modified by the authors. This involves differentiation of the organisms by their sensitivity to an antibiotic, in this case bacitracin. It is claimed that the method is adaptable to the work in small laboratories and gives a low percentage of error (3%) as compared with the serological method.

TOXOPLASMOSIS IN NEW ZEALAND

J. D. Manning and J. D. Reid
N.Z. med. J., **55,** 441, 1956

Some of the technical difficulties encountered with the dye and complement fixation tests for toxoplasmosis are discussed. After some experience had been gained in their use, these tests were found to be as reliable and reproducible as other serological procedures.

Serological evidence is presented which shows that toxoplasma infection with or without recognised symptoms, is very common. By the age of 40 over 50% of the persons tested had acquired antibodies to toxoplasma.

STUDIES ON THE EPIDEMIOLOGY OF POLIOMYELITIS IN NEW ZEALAND

A. M. Murphy and Margaret B. Brown
N.Z. med. J., **55**, 28, 1957

During the epidemic of the summer of 1955-56, thirty-eight poliovirus isolations were made. Thirty-five of these were Type 1 which seems to be of a lower order of virulence than those previously encountered.

Serological surveys carried out before and after the epidemic showed a 20% increase in the population immunity and this supports the theory that the strain had a lower virulence.

BENIGN CRYOGLOBULINAEMIA PURPURA

F. W. Gunz
Brit. J. Haemat. **2**, 95, 1956

This is a case report of a woman diagnosed as benign cryoglobulinaemia purpura. This rare syndrome is characterised by attacks of purpura accompanied by intense pain and usually provoked by exposure to cold. The abnormal globulin, cryoglobulin is believed to be responsible for the attacks of purpura. This abnormal globulin precipitates spontaneously at temperatures below 35°C. It is not known by what mechanism the constant production of this cryoglobulin is initiated or is maintained. The presence over a period of years of purpura resulting from cryoglobulinaemia, without any ascertainable primary disease, is extremely rare. This is believed to be the fourth case of benign cryoglobulinaemia purpura to be reported.

THE INFLUENCE OF BLOOD COLLECTING TECHNIQUES ON PLATELET NUMBERS DURING BLOOD STORAGE

J. F. Mustard and C. B. V. Walker
Brit. J. Haemat. **3**, 50, 1957

The authors compare the effect of different methods of blood collection on the subsequent stability of platelets during blood storage. The three methods used are (1) the standard National Blood Transfusion Service (N.B.T.S.) gravity method; (2) the vacuum bottle collection technique; (3) gravity collection using a N.B.T.S. taking set modified by replacing red rubber with latex rubber and all surfaces of the set coming in contact with the undiluted blood coated with silicone. Results show that the type of surface used in blood taking sets is an important factor in preventing platelet loss. There is a considerably reduced loss of platelets during blood collection when collecting sets with silicone-coated and plastic surfaces are used, and platelets in these bottles are more stable during storage than those in blood in which there has been a considerable platelet loss during collection.

ANTIBACTERIAL ACTIVITY OF NOVOBIOCIN AND VANCOMYCIN

R. W. Fairbrother and B. L. Williams
Lancet **2**, 1177, 1956

Tests to determine the sensitivity of the common pathogens to the new antibiotics were carried out by the dried disc technique and serial dilution method. It was shown that vancomycin and novobiocin are active against most Gram positive cocci and should be particularly valuable in the treatment of infections caused by penicillin resistant staphylococci. Little or no activity against the Gram negative bacilli, usually associated with urinary infections, was found.

THE TRANSMISSION OF STAPHYLOCOCCUS AUREUS

R. Hare and C. G. A. Thomas
Brit. med. J., **2**, 840, 1956

It is suggested that this organism is not transported from person to person by droplets or droplet nuclei but by an indirect route involving egress in nasal secretions, contamination of the skin, clothing or bedding, often by hands, release of the organisms by friction, movement, or washing and transportation to others by air currents. It is shown that some carriers can contaminate the atmosphere in their neighbourhood with much larger numbers of Staphylococcus aureus than the majority of carriers.

STAPHYLOCOCCI HARBOURED BY PEOPLE IN WESTERN HIGHLANDS OF NEW GUINEA

P. M. Rowntree
Lancet **1**, 719, 1956

None of the strains of Staphylococcus aureus isolated were antibiotic resistant. Some non-pathogenic staphylococci found in nasal swabs were resistant to antibiotics. Phage typing of the strains isolated from these primitive people showed that these were similar to those found in civilised man.

MIXED STAPHYLOCOCCAL INFECTIONS THE DEVELOPMENT OF PENICILLIN-RESISTANT STRAINS

R. W. Fairbrother
Lancet **1**, 716, 1956

The origin of penicillin-resistant strains of staphylococci is discussed in this report of investigations under natural and under experimental (*in vitro*) conditions of hospital infections. The laboratory conditions appear to be exacting, though the hypotheses drawn from the results of experiments and from clinical observations—would seem to be somewhat shaky. The article shows that penicillin sensitive strains cannot be changed in the laboratory to resistant strains except in the instances where they have originally been penicillin-resistant. The enzyme penicillinase is considered a controlling factor in mutation ability, and this is enhanced by the indiscriminate use of penicillin.

BACTERIOPHAGE TYPING OF ENTERIC PATHOGENS AND STAPHYLOCOCCI AND ITS USE IN EPIDEMIOLOGY

E. S. Anderson and R. E. O. Williams
J. clin. Path., **9**, 94, 1956

A review of bacteriophage typing and its uses in epidemiology is given.

THE PROBLEM OF STAPHYLOCOCCAL INFECTION

W. McDermott
Brit. med. J., **2**, 837, 1956

Two approaches to the problem are discussed. Firstly hospitalised patients today are on the whole less able to cope with staphylococci than was formerly the case. This means that aseptic practices not only in the operating room but especially in connexion with the ordinary "puncture" procedures should be reviewed.

Secondly they suggest the means for increasing bodily defences against staphylococci and means to restore these defences when they have been reduced.

The crux of today's staphylococcal problem lies not so much in changes in staphylococci as it does in changes in the status of their hosts.

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